بسم الله الرحمن الرحيم

University of Khartoum
The Graduate College

DEVELOPMENT AND EVALUATION OF PCR ASSAYS FOR DETECTION OF ANIMAL-DERIVED PRODUCTS IN PROCESSED FOOD AND ANIMAL FEED CONCENTRATES

A thesis submitted in accordance with the requirements of the University of Khartoum for the Degree of Doctor of Philosophy

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بسم الله الرحمن الرحيم

(وما أوتيتم من العلم إلا قليلاً)

سورة الإسراء الآية (85)

(و قل رب زدني علماً)

سورة طه الآية (114)

Dedication

Gratefully dedicated to my loving parents who brought me up; help me in education and to all brothers, sister and colleagues.

Acknowledgements

Firstly, my gratitude and thanks to my supervisor; Professor.Imadeldin E. Aradaib for his excellent supervision, advice and support. Also I would like to thank the Head Department, colleagues and members of the Department of Medicine, Pharmacology and Toxicology for their great help and assistance.

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ABSTRACT

Nested (nPCR) and semi-nested polymerase chain reaction assays for specific identification of animal-derived products in processed food and in animal feed concentrates were developed and evaluated. The mitochondrial cytochrome-b (mtcyt-b) gene was used as a target DNA for PCR amplification. Pairs of primers derived from highly conserved regions of mtcyt-b gene were used for the PCR assays in two amplification steps. For the specific identification of swine mtcyt-b gene, two pairs of primers (PSL1 and PSR2) and (PSL3 and PSR4), were used. The outer pair of primers (PSL1&PSR2) produced a 1055 base pair (bp) PCR product from swine DNA. Amplification products were visualized on ethidium bromide-stained agarose gels from 100 fg of swine DNA equivalent to 1000 copies of mtcyt-b gene. The nested primers (PSL3 and PSR4) produced a 361 bp PCR product, internal to the annealing sites of primers (PSL1 &RSR2). The nested amplification confirmed the identity of the primary amplified PCR product and increased the sensitivity of the PCR assay. The nested PCR with ethidium bromide-stained

agarose gels detected the amount of as little as 0.001 fg of DNA (equivalent to a single copy of Swine-mtcyt-b gene). The specificity studies indicated that neither the primary 1055 bp nor the nested 361 bp PCR products were detected from DNA extracted from a variety of other animal species including, sheep, goat, cattle, deer, camel, horse, donkey, chicken and fish. Application of this nested PCR to processed food including, fresh pork, smoked ham, marinated pork, canned luncheon, pet's food, poultry feed resulted in amplification of the swine specific PCR products.

Semi-nested PCR, a similar technique was also used in this study. It revealed the same results as nPCR when applied to cattle mtcyt-b gene using two pairs of primers (RSL1 and CSR2) and (CSL1 and CSR2) which produced 386bp and 283bp PCR products, respectively.

ملخص الأطروحة

في هذه الدراسة تم تطوير وتقويم تقنية التفاعل التبلمري المتسلسل الشبكي (nPCR) وشبه الشبكي للتعرف على اللحوم والمنتجات الحيوانية في الأطعمة والأعلاف و المركزات المستوردة. وقد أستخدم فيها جين السيتوكرم (ب) الميتوكونديري (mtcyt-b) كحمض نووي مستهدف للتكبير.

تمت التقنية على مرحلتين لتكبير الجين المستهدف(mtcyt-b) إحتوت كل منهما على أزوج من البادئات المصممة من أكثر الأجزاء ثباتاً من الجين المستهدف. فلتكبير جين الخنازير أستخدم زوجان من البادئات، الزوج الأول الخارجي وسمّي (PSL1 وPSL2) وبواسطته تم الحصول على منتج بحجم 1055 زوج قاعدي من تركيز نهائي للجين المستهدف يصل حتى 100 فيمتوجرام أي ما يعادل 1000 نسخة من الجين. وبإستخدام الزوج الآخر الداخلي (PSL3 وPSL4) تم الحصول منتج بحجم 361 زوج قاعدي وزادت حساسية التفاعل لتصل إلى تركيز نهائي من الجين المستهدف يصل إلى 0.001 فيمتوجرام وهو ما يعادل نسخة واحدة من الجين المستهدف.

إضافة للحساسية العالية أوضحت التقنية خصوصية عالية للجين الخاص بالخنازير فلم يتم بواسطتها التكبير الأساسي أو الشبكي لأي من جينات الحيوانات الأخرى سواءً من المجترات كالأبقار والأغنام والماعز والغزلان والجمال أو ما عداها من الحيوانات كالخيول والحمير والدجاج والسمك. كما إن تطبيق تقنية التفاعل التبلمري المتسلسل الشبكي على المنتجات المصنعة والمعالجة مثل اللحم المشوي والمدخن والمتبل والمعلب وطعام القطط والكلاب وعلائق الدواجن والأبقار أثبت كفاءة عالية في كشف جينات الخنزير الخاصة.

طبقت أيضاً في هذه الدراسة تقنية مشابهة وهي تقنية التفاعل التبلمري المتسلسل شبه الشبكي. وقد طبقت على جينات الأبقار بنفس الخطوات السابقة حيث أستخدم زوجان من البادئات

هما (RSL1 وCSR2) و(CSL1 وCSR2) واللذان ينتجان منتجات للتكبير بطول 386 و 283 زوج قاعدي على التوالي. وقد أفرزت نتائج مشابهة لتلك التي حصل عليها في تقنية التفاعل التبلمري المتسلسل الشبكي.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1-1 Introduction:

Consumers' health and satisfaction is very important in food industry, because of which authorities put regulations to control the quality of food industry. Consumers' health is reached by either detecting the harmful materials and chemicals or by maintaining the minimal recommended amount of food or feed additives and preservatives. While the satisfactory of consumers is obtained by tracing the origin of materials used in processed food. So consumers demand quality products that are well labeled. Also in many African and third world countries (including Sudan), regulatory officials required and relied on labeled products for this verification. However, fraudulent or unintentional mislabeling still exist and may not be detected, resulting in a poor-quality product.

It is well-known documented that breeding of pigs and consumption of pork is common in some countries. This is verified by the presence of more than five farms for breeding pigs in Khartoum State only. However, some populations such

as vegetarians, Muslims and Jewish prohibit and do not desire pork consumption. This population demands methods to detect pork in food while identification of pork in products has several important applications in the food industry, not only to detect, falsely labeled products but also for economic, religious and health reasons.

Additionally people are nowadays concerned about meat in general because of disease as e.g. bovine spongiform encephalitis (BSE), variant Creutzfeld Jacob Disease, Foot-and-Mouth Disease or Rift Valley Fever. The globalization of the market, including food production and trading, has lead to new problems the food control is confronted with. Food and feed can come from every corner of the world just following a maximization of the profit. Thirst for profit and globalization were main reason for the spreading of BSE. Animal species from all over the world find their way in our foods.

Fresh pork is a protein in nature, which could be detected by immunologic assays. However, it is rather difficult to identify cooked, marinated or dried pork by immunological

assays due to protein denaturing. In Sudan, no reliable assays exist for detecting the presence of pork in processed food.

For all the previously mentioned reasons, the development of a molecular diagnostic technique for identification of biomaterials from complex sources would be advantageous in a variety of circumstances including comparative genomic and investigative forensics.

Therefore, nucleic acid hybridization and sequencing have been successfully applied for animal species identification in human food and animal feed.

1-1-1 Polymerase Chain Reaction (PCR):

The polymerase chain reaction (PCR) is a primer- mediated enzymatic amplification of specifically cloned or genomic DNA (Lori *et al*, 1997). In other words, it is an alternative technique to cloning and isolating a DNA sequence with which can selectively amplify a single copy of a desired DNA sequence present in complex mixture of DNA molecules. This technique was invented by the American scientist Kary Mullis in 1983.

1-1-2 Primers:

They are short oligonucleotides (dNTPs) which designed as start points of DNA amplification with PCR (Lori *et al*, 1997). Primer must provide a free hydroxyl group (-OH) at the 3′ to which polymerase enzyme can add dNTPs to extend the sequence.

1-1-3 Taq DNA Polymerase:

It is a recombinant enzyme produced by *E.coli* from DNA polymerase gene of the thermophilic bacteria *Thermus aquaticus* (Lori *et al*, 1997).This enzyme is stable at high temperatures used in PCR reaction while other eukaryotic and prokaryotic polymerases are damaged in high temperatures. This enzyme is needed to extend the sequence amplification from the primers ends.

1-1-4 Buffers:

PCR buffer for catalyzing Taq DNA polymerase enzyme consists of 50 Mm of KCl and 10 Mm of Tris-HCl(Lori *et al*, 1997). This buffer is stored at room temperature and pH 8.3.

The buffer adds ionic strength and provides the reaction with the buffering capacity needed. Also magnesium chloride (MgCl) is needed as a very essential metal ion cofactor. The concentration of MgCl is varying from 1.5-4.0 Mm depending on dNTPs, primers and template concentrations.

1-1-5 Gel Electrophoresis buffers:

This technique is used for visualization of PCR products. The main buffer is Tris Borate EDTA (TBE) buffer which consists of 89 Mm Tris-borate and 2 Mm EDTA. This buffer is provided as 10X concentration stock buffer. Then used in both preparing the agarose gel and for running in the electrophoresis apparatus (Lori *et al*, 1997).

Objectives of the study:

In Islamic religion pork and swine derived products are prohibited to be consumed or used in other purposes. So it is very important and useful to detect pork and swine-derived products in animal feed, foodstuff and imported products. This is the main objective of this study in which we intended to find

an alternative method to the old immunochemical methods or develop the similar method to be more specific, rapid and reliable. We used nucleic acids amplification technology, commonly known as Polymerase Chain Reaction (PCR), as a rapid, sensitive and specific assay for detection of pork or swine-derived products and other animal derived products in processed food, animal feed and biocomplex materials.

1-2 Literature Review

1-2-1 Detection of animal derived products

Detection of animal derived products was firstly used to detect alteration in animal products. This was become the most important when special case imerged; Bovine Spongiform Encephalopathy (BSE) or mad cow disease while it was known the fact that the causative agent of this disease is transmitted by animal byproducts fed to animals (Allman et. al.,1993, Aradaib, 1998a-b).

1-2-1-1 Detection of animal-derived products using Immunochemical assays:

Immunologic assays such as enzyme-linked immunosorbent assay (ELISA) were used to identify animal proteins in commercial animal feed. (Kim et al, 2004) used monoclonal antibodies in ELISA to detect h-caldesmon of bovine smooth muscles in animal feed. Also (Chen et al, 2004) used sandwich ELISA to identify skeletal muscles protein known as troponin I in feedstuff. They stated the possibility to detect 5.0 and 4.0 ng/ml of the extracted bovine and ovine troponin I respectively.

Cooked porcine skeletal muscles proteins were detected by (Chen and Hsieh, 2000) using ELISA. They reported the detection of 0.5% of porcine proteins in meat mixtures but only those found in skeletal muscles not the cardiac muscles, smooth muscles, blood, and nonmuscle organs.

(Martin D.R. et al, 1998) reported the usage of ELISA and radial immunodiffusion (RID) to detect pork alteration. They detected concentrations between 1-75% and 0.0625-2 % of porcine proteins by ELISA and RID respectively. They also reported the identification of swine albumin in spiked meat

samples with the sensitivity of 1% and 3-5% for ELISA and RID respectively.

Earlier, (Hsieh et al, 1998) stated the possibility of detection of pork, beef, hogs, sheep, horse, and deer meat though heated to 100º C for 15 minutes with ELISA. However, they failed to detect poultry, raw meat or less than 0.5% of the extracted proteins.

Poultry and pork cooked and canned meats were also tested by ELISA (Berger, 1988). They detect 126ppm of turkey and chicken proteins and 250ppm of pork proteins. They used the heat resistant monoclonal antibodies against skeletal muscles.

A comparative study was done by (Allmann et al, 1993). They compared between the immunochemical and molecular methods in assuring safety and quality of food. They found the later more rapid and sensitive.

1-2-1-2 Detection of animal-derived products using PCR:

This method depends on identification of the nucleic acid as said in section (1-1-1). Species specific DNAs were mostly used to identify animal-derived product. Lanzilao *et al.,*

2005) reported that species identification plays an important role in food allergy prevention and food substitution detection that can reduce the commercial value of a product. For these reasons many molecular methods have been developed to determine species origin among them, polymerase chain reaction (PCR). Based methods were successfully applied to a processed and unprocessed food stuffs. The European community ban on use of meat and bone meal in ruminant feed, as consequence of the spread of bovine spongiform encephalopathy in Europe, has promoted a number of investigations about the possibility of detecting animal tissues in feed stuff (Bottero *et al.,* 2003). Wang *et al.,* (2003) developed PCR assay for detection and identification of bovine derived materials in imported animal feeds and food. Hsieh *et al.,* (1998) detection of species adulteration in ground meat products is important for consumer protection and food labeling law enforcement.

Rajapaksha *et al*., (2002) reported polymerase chain reaction (PCR) assay to differentiate meat of ceylon spotted deer, ceylon hog deer, ceylon samb hur , and barking deers from meat of cattle , goat, buffalo, pig, dog and sheep. Quantitative

estimates are important to establish whether pork adulteration in ground beef pate is accidental or intentional. A PCR producer has been developed and evaluate to quantity pork in heated and nonheated meat and pates as described by Calvo *et al.,* (2002). Calvo *et al.,* (2001) also developed and evaluated a PCR producer to detect pork in heated and non heated meat, sausages, canned food, cured products and pates using a faster, more specific and more sensitive method than other previously described. They concluded that because some fraudulent or unintentional mislabling occurs that can be undetected resulting in lower quality pate, and because some population groups for philosophical or religious reasons do not wish to eat meat from certain species a new producer was developed and evaluated to detect pate species composition.

Meyer et al., (1995) described polymers' chain reaction (PCR) technique to detect meat species identification in marinated and heat-treated or fermented product and to the differentiation of closely related species.

1-2-1-3 Detection of Swine-derived products using PCR

Meyer *et al.,* (1994) developed a new method for the specific, sensitive, and semi quantitative detection of pork (*Sus scrofa*) in heat-treated meat products, by using a DNA-binding resin and subjected to polymerase chain reaction (PCR) analysis. The test detected pork in fresh or heated meat mixtures of pork in beef at levels below 2%. (Montiel-Sosa *et al.,* 2000) designed the highly species-specific primers for pork D-loop mtDNA. These primers and restrictive PCR amplification conditions has improved a reliable and rapid method for detecting a PCR-amplified 531 bp band from pork. It has been proved useful for detecting both pork meat and fat in meat mixtures, including those dry-cured and heated by cooking.

Calvo *et al.,* (2001) developed and evaluated a PCR procedure to detect pork in heated and nonheated meat, sausages, canned food, cured products, and pates using a faster, more specific, and more sensitive method. Pair of primes was synthesized to confirm the effectiveness and specificity of this fragment, 55 pig blood DNA samples (from different breeds) were tested and positive results were obtained. By using this

method, up to 0.005% pork in beef and 1% pork in duck pate using 30 and 20 PCR amplification cycles respectively was detected. It is a very fast method, because 1% pork contamination can be detected with 20 PCR cycles. (Calvo *et al.,* 2002) developed a PCR procedure and evaluated to quantify pork in heated and non heated meat and pates by densitometry using a specific and sensitive repetitive DNA element. Thirty, twenty-five, and twenty PCR cycles were carried out to find the best standard curve and correlation between pork content and band intensity. Twenty cycles showed the best results, quantifying degree contamination up to 1% pork in beef (heated and non heated) and pork in duck pate with a minimum error. Finally, fraud was found in commercial pates. (Bottero. *et al.,* 1998) reported that the effectiveness of detection of beef and pig meat, which had been subjected to severe heat treatment in foods including dried skim milk, homogenized in fat food and canned meat. DNA analyses were used to confirm that this technique is suitable for reliable identification and labeling of food components.

Meyer *et al.,* (1995) reported that the polymerase chain reaction (PCR) technique was applied to meat species identification in marinated and heat-treated or fermented products and to the differentiation of closely related species. DNA was isolated from meat samples by using a DNA-binding resin and was subjected to PCR analysis. Primers used were complementary to conserved areas of the vertebrate mitochondrial cytochrome b (cytb) gene and yielded a 359 base-pair (bp) fragment. Analysis of sausages indicates the applicability of this approach to food products containing meat from 3 different species. The PCR-RFLP analytical method detected pork in heated meat mixtures with beef at levels below 1%, and the method was confirmed with porcine- and bovine-specific PCR assays by amplifying fragments of their growth hormone genes. (Sun and Lin 2003) applied a method of fluorescent Polymerase Chain Reaction-restriction fragment length polymorphism (PCR-RFLP) as an analytical and quantitative tool for meat identification, an oligonucleotides primer pair was designed to amplify the partial sequences within the 12S ribosomal RNA (12S rRNA) gene of mitochondrial DNA

from porcine, caprine, and bovine meats. Using fluorescence sensor capillary electrophoresis, the species-specific DNA fingerprints of pork, goat, and beef were generated by restriction enzyme digestion following a fluorescence-labeling PCR amplification. Species identification was conducted on the meat mixtures. The reliably semi quantitative levels were below 1% for binary mixtures of pork, goat, and beef. Cooking and autoclaving of meats did not influence the generation of the PCR-RFLP profiles or the analytical accuracy. (Rajapaksha *et al.*, 2002) developed a polymerase chain reaction (PCR) assay to differentiate meat of some species of deer from meat of cattle, goat, buffalo, pig, dog and sheep. A set of primers was designed according to the sequence of the mitochondrial cytochrome b gene of *C. elaphus canadensis* using PCR amplification about 450 bp bands was observed for all four animal species. A band of 649 bp sizes was observed for all animal species when DNA was amplified with the universal primers and that indicated the presence of mitochondrial DNA in the samples. Further, the results indicated that this technique was sensitive enough to

differentiate rotten meat, at least 5 days after the killing of an animal.

Calvo *et al.,* (2001) used the RAPD method to generate fingerprint patterns for pork, chicken, duck, turkey, and goose meats. Ten DNA samples from pork, chicken, turkey and duck meats were tested to confirm the effectiveness and specificity. Specific results for each species were obtained by the RAPD method sensitivity of the method was studied by DNA dilution in each species detecting as little as 250 pg of DNA. (Dalmasso *et al*., 2004) used the species of ruminant, poultry, fish and pork. Primers were designed in different regions of mitochondrial DNA (125 + RNA, TRNA val and 165 rRNA). The primers generated specific fragments of 104-106, 183, 220 – 230 and 290 bp lengths for ruminants, poultry fish and pork, respectively. The detection limit was 0.004% for fish primers and 0.002% for ruminants, poultry and pork primers. (LIhak and Arsalan, 2003) identified cattle, sheep, goat and wild pig meats by using Random Amplified polymorphic DNA (RAPD) techniques, with a 10 base primer (CGCCCTGGTC) to determine meat of cattle, sheep, goat and pig origin in turkeys.

(Rodriguez *et al* 2004) were designed common primer on a conserved DNA sequence in the mitochondrial 125 ribosomal RNA gene (rRNA) and reverse primers were designed to hybridize on species – specific DNA sequences of each species considered. The allowed clear species identification analysis of experimental meat mixture demonstrated that the detection limit of the easy was 1% (wt/wt) for each species analyzed. (Obrovska et al., 2002) extracted the DNA from the respective meat species and subjected to the PCR analysis using a mixture of seven primers together in the appropriate ratios and identified using one PCR reaction. The design of the forward primer was based on the known DNA sequences of the cytochrome b mitochondrial gene; the reverse primers were designed based on species-specific sequence depending on the meat species. The resulting fragments at the 157, 227, 274, 331, 398, and 439 bp levels corresponded to goat meat – chicken, beef, multon pork and respective species of both heat – processed and non-heat processed meat.

Lanzilao *et al* (2005) developed an updated PCR RFLP (restriction fragment length polymorphism) method of the cyTb

gene for the identification of the 4 species of main interest in the dairy industry Bos, ovis, capra, Bubalus). The comparative analysis of the 92 cyTb sequences available in the database belonging to the 4 species allowed identification of 2 highly conserved regions, which were used to design 2 oligonucleotides for the PCR amplification of a 275 base pair (bp) cyTb fragment. (Bottero *et al*., 2003) studied on vertebrate primers, designed in the 165 rRNA gene of mitochondrial DNA. These primers were able to amplify fragments that contained between 234 and 265 bp. The fragments were specific for bovine, porcine, goat, sheep, horse, rabbit, and chicken trout. The primers were used in a PCR assay applied to fire samples of meat and blood meals of different species and subjected to severe rendering treatments (134.4 to 141.9 degree C and 3.03 to 4,03 bar for 24 minutes). The assay proved to be rapid and sensitive (detection limit 0.0625%) it can be used as a routine method to detect animal- derived ingredients in feedstuff. (Krcmar and Rencova 2003) tested the specificity of the primers for PCR using samples of DNA of other vertebrates. The method allows the detection in concentrate mixtures of 0.01% of the

Target species derived material. The identity of a sample containing 0.1% of bovine, ovine, swine, and chicken meat- and – bone meal has further been confirmed by sequencing. (Rodrigues 2003) used qualitative PCR for detection of chicken and pork adulteration in goose and mule duck. The design of species – specific forward primers together with a reverse universal primer, allowed the generation of amplicons of different lengths in each species. The different sizes of the species specific amplicons, separated by agarose gel electrophoresis – allowed clear identification of the presence chicken and pork in goose and mule duck foie gras with a detection limit of 0.2% (w/w). The technique could be used in inspection programmed to enforce babbling regulation of foie gras and other meat products. (Wang-chong *et al.,* 2000) used Diluted of whole pig blood, directly analyzed by the agarose gels by ethidium bromide staining. The results show that the band using 2% whole blood is rather weak, 8% blood inhibited the PCR; 4 or 6% whole blood was the best. The PCR products were digested with the enzyme Hha. The bands were distinct and bright. (Calvo et al., 2002) used a specific and sensitive method

to confirm the effectiveness and specificity of beef- and bovine-derived materials using PCR amplification to detect the degree of contamination up to 0.01% raw beef in pork. Further more feed components intended for cattle nutrition can be checked. (Partis *et al.,* 2000) found that the technique was used to generate DNA fingerprints for 22 animals' species by amplifying a 359- bp region within the cytochrome b mitochondrial gene. Pig was preferentially amplified and dominated over the other species tested.

Biase *et al.,* (2002) obtained high molecular weight DNA of good quality, shown by agarose gel and amplification of two DNA fragments. 605 and 891bp, by PCR spectrophometric analysis of DNA concentration showed variation among the DNA from different tissues. (Mercier et al., 1990) proved PCR method to work not only on fresh blood samples but also frozen blood samples stored several months at – 20°C. Up to 900 bp from frozen whole blood samples were successfully amplified fragment. Aliquots of amplified fragment from PCR performed either on purified DNA or blood were analyzed directly on agarose gels by ethidium bromide. No difference was noticed

between purified and blood samples for all the different amplified sequences tested. (Chen and Hsich 2000) reported that an enzyme- Linked immunosorbent assay (Elisa) using a monoclonal antibody to a porcine thermal. Stable muscle protein was developed for detection of pork in cooked meat products. The detection limit was determined as 0.5% (w/w) pork in heterogonous meat mixtures. (Meryer 1996) found that the presence of Soya deoxyribonucleic acid (DNA) from several Soya protein concentrations was determined with two pairs of specific oligonucleotides yielding a 414 bp (base pair) fragment and an internal 118-bp fragment amplified from the soya lection le T gene. The test detected DNA from textured soya protein concentrates in meat products at level or 1% and was confirmed by a commercially available enzyme – linked immunosorbent assay (ELISA). (Zehner *et al.,* 1998) said that to identify common animal species by analysis of the cytochrome B gene (981 bp out of 1140 bp) in humans. Selected mammals and birds using the same specifically designed primers species-specific RFLP patterns are generator by co-restriction endo nuclease ALU1 and Nco 1.The RFLP patterns obtained are conclusive

even in mixtures of two or more species, the method was applied to forensic stomach contents and bone sample was successfully identified.

(Wolf *et al.,* 1999) found that the basis of amplification of specific part of the mitochondrial genome (IRNA GLU)/ (Cytochrome b) using the PCR to obtained 464- bp. Long PCR products were cut with different restriction endonuclease (RE) resulting in species – specific restriction fragment length polymorphism (RFLP). (Chen *et al.,* 2005) established a sensitive PCR method based on amplification of a specific DNA fragment for the identification of camel (*Camelus*) materials. The species- specific primers pair L1 & 3/H372 was designed based on the nucleotide sequence of the mitochondrial cytochrome b gene. An expected 208 base pair fragment was amplified from camel material. (Nemeth *et al.*, 2004) used an optimized DNA extraction protocol for animal tissue coupled with sensitive PCR methods to determine whether trace level of feed- derived DNA fragments, plant and /or transgenic are detectable in animal tissue sample including dairy milk and sample of muscle (meat) from chickens, swine and beef steers.

Assays were developed to detect DNA fragments of both the high copy number chloroplast- encoded maize rubisco gene (r bcl) and single copy nuclear-encoded transgenic elements (P 355 and amon 810- specific gene fragment). (Yancy *et al.,* 2005) developed a rapid PCR based analytical method for detection of animal-derived materials in complete feed using a commercially available DNA forensic Kit for the extraction of DNA from animal feed. A sensitive method was developed to detect as little as 0.3% bovine meat and bone meal in complete feed in less than 8 hours of total assay time. (Cespedes *et al.,* 1999) used PCR amplification of the nuclear 5s rRNA gene for the identification of sole and green land halibut by simple agarose gel electrophoresis. Two distinguishable band patterns for both species were appeared. (Kocher *et al*., 1989) To amplify homologous segments of mt DNA from more than 100 animals species. Were using unpurified mt DNA from nano gram samples of fresh specimens and micro gram amounts of tissues preserved for months in alcohol or decades in the dry state. (Sullivan et al., 1992) extracted degraded DNA from bone fragment and a necrotic skin sample and amplified at 2 hyper

variable segments within the mitochondrial non- coding region using 2 rounds of nested PCR. (Skarpeid *et al,* 1998) reported that based on intensity profiles from isoelectric focusing or water – soluble proteins in mixtures or ground meat samples contains various amounts of beef, pork and turkey meat were analyzed by isoelectric focusing in immobilized PH. Gradients resulting gel profiles were analyzed by multivariate regression allowing the determination of sample composition errors close to 10%. Hsieh *et al*, (1998) said that detection of species adulteration in ground meat products is important for consumer and were used in an enzyme- linked immunosorbent assay (ELISA) for rapid detection of any cooked mammalian meats in cooked poultry products. Soluble muscle proteins extracted from cooked pork (heated at 100 degree C for 15 minutes) were used as the antigen to immunized mice for developing the MAb. Random amplified polymorphic DNA polymerase chain (RAPD PCR) fingerprints in forensic species identification. Random amplified polymorphic DNA polymerase chain (RAPD PCR) fingerprints in forensic species identification. (Wilson *et al.,* 1995) developed techniques for extracting, amplifying and

directly sequencing mitochondrial DNA (mt DNA) from human hair shafts. DNA was extracted with organic solvent and purified by titration. The mTDNA sequence from the hair shaft match the mt DNA sequence from blood samples taken from the same donor. (Gao 2004) reported that the amplified canis species PCR product was a 213 bp band from the D – loop DNA fragment of mitochondria, a high copy gene that should improve the possibility of amplifying. The specificity of this method was confirmed by 8 canis blood DNA samples (Jennings *et al*, 2003) used highly sensitive, pork loin samples to analyze the presence of fragments of transgenic and endogenous plant DNA and transgenic protein from animal fed meal prepared from conventional or glyphosate. Tolerant Roundup ready (RR) soybeans.

Total DNA was extracted from the samples and analyzed by PCR followed by southern blot hybridization for the presence of a 272 b p fragment of the CP4 epsps-coding region. (Lahiffs *et al.,* 2002) detected the bovine DNA extracted from meat and bone meal (mBm) samples. PCR primers were used to amplify a 271 bp region of the mitochondrial ATP are 8. ATPase 6 gene-

DNA extracted from blood and with PCR products generated from genomic DNA extracted from single – species laboratory scale rendered mBm samples.

Bottero *et al.,* (2003) studied on vertebrate primers, designed in the 165 rRNA gene of mitochondrial DNA. These primers were able to amplify fragments that contained between 234 and 265 bp. The fragments were specific for bovine, porcine, goat, sheep, horse, rabbit, and chicken trout. The primers were used in a PCR assay applied to fire samples of meat and blood meals of different species and subjected to severe rendering treatments (134.4 to 141.9° C and 3.03 to 4.03 bar for 24 minutes). The assay proved to be rapid and sensitive (detection limit 0.0625%) it can be used as a routine method to detect animal- derived ingredients in feedstuff.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Collection and preparation of samples:

2.1.1 Blood samples

Blood samples were collected in clean sterile vacutainers, containing ethylene diamine tetra acetic acid (EDTA), from pigs (positive controls) and from different animal species including cattle, sheep, goats, camels, deer, chickens, horses, donkeys, fishes.

Pig blood samples were collected from three pig farms; Metal Factory Farm, Western Omdurman Church Farm and Idbabiker Church Farm. While the other species samples were collected from Khartoum University Dairy Farm and from the animals attended the Veterinary Teaching Hospital.

The blood samples then centrifuged in bench centrifuge (Hettich Zentrifugen, D-785320, Tuttiligen, Germany)(Appendex-Fig1) in order to separate the buffy coat which is rich in white blood cells and used for extraction of total genomic and mitochondrial cytochrome-b (mtcyt-b) DNA. The extracted DNA was used as a target DNA for PCR amplification.

2.1.2: Pork samples:

Tissue samples were obtained during slaughtering from the previously mentioned farms. Also some of the samples were gathered in Soba Hospital when they used pigs as a model in their surgery module.

2.1.3: Animal feed and Concentrates samples:

Animal feed and concentrates were obtained from the suppliers in the local market.

2.1.4: Foodstuff samples:

Some of the food stuff samples were obtained from the local market suppliers while others specially swine derived ones were brought from Germany such as smoked pork and tuna.

2.2: Extraction of Nucleic Acid:from blood samples:

2.2.1: Phenol Method:

2.2.1.1. Preparation of reagents and buffers:

In this method, the main reagent was prepared from the mixture of phenol, chloroform and isoamylalcohol at the ratio of 25:24:1 respectively (Ibrahim *et al* 2004) The mixture then stored in room temperature to be used later to extract the nucleic acid in the standard method.

For destruction of cellular materials numbers of buffers were prepared. Sodium acetates buffer was prepared by dissolving 3.0 moles of the salt in 1 litre of distilled water to reach the concentration of (3M) (Appendix 3) and the pH was adjusted to pH 5.0. Then, half volume of the previously prepared buffer was diluted to reach (0.1 M) (Appendix 5) and pH was also adjusted to pH 5.0. Trisodium citrate buffer (tris buffer) was prepared by dissolving 0.1 mole of the salt in 1 litre of distilled water to reach the concentration of (0.1M) and the pH was adjusted to pH 8.0(Appendix 1-2). Sodium dodecyl sulphate (SDS) was prepared with 10% concentration (Appendix 8).

Sodium acetates buffered phenol and Tris buffered phenol were prepared to be used in the classical method. They were prepared by adding equal volumes of either sodium acetates buffer (pH 5.0) or Tris buffer (pH8.0), then shacked very well and water bathed on 56°C for 10 minutes. The supernatant was poured off and added another equal volume of either buffers. This was repeated twice. In the third time without pouring off the supernatant, the working solution was ready and stored in room temperature (Appendix 6-7).

2.2.1.2 First Method:

8.7 µl of (3M) sodium acetate (pH 5.0), 250 µl of (0.1M) sodium acetate (pH 5.0), 50 µl 10 % SDS, 2.5 µl of proteinase K enzyme (20 mg/ml) and 500 µl of blood samples (buffy coat) were added to 1.5 ml eppendorf tube and gently mixed and vortexed on the vortexing machine (Janke &Kunkel,GmbHu.CoKG, Germany)(appendix-Fig2) The mixture was further incubated over ice for 5 minutes and at 37ºC for other 10 minutes in order to activate trypsine to digest cells. After gentle vortexing, the mixture was incubated at 60ºC for 3-

4 minutes to destroy Proteinase K. 560µl of the extraction reagent (phenol, chloroform, isoamylalcohol) were added to the mixture, vortexed, incubated at 60ºC for 2-3 minutes and then centrifuged on the microcentrifuge (Appendix-Fig3) at the speed of 12000 rounds per minute (rpm) for approximately 2 minutes. The supernatant then collected and the nucleic acid (DNA in this case) was precipitated with 1 ml of cold ethanol by gentle mixing or vortexing followed by centrifugation on the microcentrifuge at maximum rpm for 15 minutes. After that, ethanol was pipetted off without disturbing the DNA pellet at the bottom of the tube then placed in 70ºC for at least 30 minutes. Further, the DNA pellets were resuspended in 100 µl of double distilled water (dd H_2O), stored in freezer and used as target DNA in PCR (Ibrahim A. et al 2004).

2.2.1.3 Second Method:

Beside sodium acetate, Tris and SDS buffers, two other reagents were used in this method. Tris buffered phenol and sodium acetate buffered phenol.

Whole blood was used for extraction of mtcyt-b DNA twice. Firstly, using the saturated phenol with (0.1 M) sodium acetate buffer (pH5.0). Then, with the saturated phenol with (0.1 M) trisodium citrate buffer (pH 8.0).

8.7 µl of (3M) sodium acetate (pH 5.0), 250 µl of (0.1M) sodium acetate (pH 5.0), 50 µl 10 % SDS, 2.5 µl of proteinase K enzyme (20 µg/ml) and 500 µl of blood samples were added to 1.5 ml eppendorf tube and gently mixed and vortexed. The mixture was further incubated over ice for 5 minutes and at 37ºC for other 10 minutes in order to activate trypsine to digest cells. After gentle vortexing, the mixture was incubated 60ºC for 3-4 minutes to destroy proteinase K. 560µl of sodium acetate saturated phenol were added to the mixture, vortexed, incubated 60ºC for 2-3 minutes and then centrifuged on the microcentrifuge (12000 rpm) for approximately 2 minutes.

The supernatant then collected and the DNA was extracted for the second time using 560µl of tris buffer saturated phenol, vortexed, incubated at 60ºC for 2-3 minutes and then centrifuged on the microcentrifuge (12000 rpm) for approximately 2 minutes.

The DNA was precipitated with 1 ml of cold ethanol by gentle mixing or vortexing followed by centrifugation on the microcentrifuge at maximum rpm for 15 minutes. After that, ethanol was pipetted off without disturbing the DNA pellet at the bottom of the tube then placed in 70ºC for at least 30 minutes. Further, the DNA pellets were resuspended in 100 µl of double distilled water (dd H2O), stored in freezer and used as target DNA in PCR.

2.2.1.2: Commercial Kits

Whole blood was used for extraction of mtcyt-b DNA using a commercially available QIAamp blood kit (QIAGEN Inc. Chatsworth, Canada)(Appendix-Fig4) according to the manufacturer's instructions. In details, 200 µl of whole blood, 20 µl of proteinase K enzyme stock solution, and 200 µl of lysing buffer (LA Buffer) were pipetted into 1.5 ml eppendorf tube and the mixture was vortexed and incubated at 60ºC for 10 minutes. 200 µl of absolute ethanol were added to the sample and mixed by vortexing. The mixture then was transferred to the QIAamp spin column, and was placed in a clean 2ml

collection tube and centrifuged at 8000 rpm for 1 minute. The QIAspin column was washed firstly with 500 µl of washing buffers 1 (AW1) at the same previously mentioned centrifugation speed and rewashed using washing buffers 2 (AW2) at speed 12000 rpm centrifugation speed for 3 minutes. The QIAamp spin column was then placed in a clean 1.5 ml eppendorf tube and the DNA was eluted with 200 µl of double distilled water preheated at 70ºC. Maximum DNA yield will be obtained by spinning at 12000 rpm for 1 minute after remaining for 1 minute in the room temperature. The DNA concentration was determined by spectrophotometer at 260-wave length. Five microliters of the suspended nucleic acid will be used in the PCR amplification.

2.2.2: Extraction of Nucleic Acid from fresh and processed pork:

Processed pork included cooked, smoked, marinated, luncheon and microwave-cooked pork. These samples were treated by freezing and thawing, and finally incubated at 70° C for 10 minutes to lyse the tissue contents. The insoluble

components were discarded by spinning at 8,000 rpm for 1 minute at room temperature. 200 µl of the supernatant were used to extract mtcyt-b DNA using phenol either methods or QIAamp blood kit as previously described. (Aradaib *et al*, 1998a)

2.2.3: Extraction of Nucleic Acid from foodstuff animal feed concentrates:

The animal feed and feed concentrates samples were obtained from commercial distributers in the market. These samples included dog food, pet food and commercial cattle feed and concentrate. Before extraction, the feed samples were grind into fine particles using molder and bistle.

0.5 gm of the feed concentrate was transferred to 1.5 eppendorf tube for DNA extraction. 200 µl of digestion buffer (50 µl of 10% SDS, 5 µl of 20 mg/ µl of protinase K and 130 µl of 0.1 M Tris buffer pH 8.0) were added to the eppendorf tube containing the animal feed. The feed concentrate was mixed by vortexing and incubated at 37ºC for 1 hour and then at 70ºC for 1 hour. The insoluble component of the food sample was discarded by spinning at 6000 rpm for 1 minute at room temperature. Two

hundred µl of the supernatant was used for extraction of swine DNA using the QIAamp blood kits or phenol either methods as described above (Aradaib I.E. et al, 1998a).

2.3 Selection of the primers for PCR amplification:

2.3.1 Swine primers

In this study, three pairs of oligonucleotide primers were selected from the published sequences of the swine mitochondrial Cytochrome-b gene (mtcyt-b) and used in these PCR assays (Randi et al., 1996). While in the preliminary stages a pair of primers Pig Specific Left1 (PSL1) and Pig Specific Right4 (PSR4) was used. The primer PSL1 included bases 63-84 of the positive sense strand (5)-CCC AGC CCC CTC AAA CAT CTC A. While the primer PSR4 included the bases 566-588 of the complementary strand (5): ATG TAC GGC TGC GAG GGC GGT AA. The amplification of mtcyt-b using primers PSL1 and PSR4 resulted in a 525 base pairs (bp) product. The report of these results was published in the Pakistan Journal of Biological Sciences (2005), volume 8, pages from 501 to 504.

In the main study, oligonucleotides primers (PSL1) and Pig Specific Right2 (PSR2) were selected for the synthesis of swine-specific primary PCR product. The primer PSL1 gave the positive sense strand. PSR2 included bases 1092-1117 of the complementary strand (5)-CGA TGA TGC TAG TGA TTG GTA TCA AT. The primary PCR amplification using primers PSL1 and PSR2 resulted in amplification of a 1055 bp PCR product from swine mtcyt-b DNA.

For nested PCR identification of swine DNA, a pair of primer Pig Specific Left3 (PSL3) and (PSR4) was used in a nested format. The nested primers were designed based on the same swine mtcyt-b DNA sequence, internal to the annealing sites of PSL1 and PSR2. PSL3 included bases 228-251 of the positive sense strand (5)-ATG AGT TAT TCG CTA TCT ACA TGC. PSR4 gave the complementary strand (5)-ATG TAC GGC TGC GAG GGC GGT AA. The nested primers resulted in amplification of a 361 bp PCR product, internal to the annealing sites of primers PSL1 and PSR2.

2.3.2 Cattle primers:

Two pairs of primers were selected from a conserved region of cattle mtcyt-b gene which published by (Irwin D.M. et al, 1991).The first pair consists of Ruminants Spcific Left1 primer (RSL1) and Cattle Specific Right2 primer (CSR2) which was used to synthesize the primary bovine-specific PCR product. RSL1 included bases 12-40 of the positive sense strand (5)-CCC AGC CCC CTC AAA CAT CTC A. CSR2 included bases 357-376 of the complementary strand (5)-GGCTATTACTGTGAGCAGA. Using of this pair of primers (RSL1 and CSR2) in PCR assay resulted in 386 bp PCR product from bovine mtcyt-b DNA.

Another pair of semi-nested primers was used for the specific identification of bovine mtcyt-b gene. Cattle Specific Left1 primer (CSL1) included bases 93-111 of the positive sense strand (5)-GAATTTCGGTTCCCTCCTG. Using primers (CSL1 and CSR2) in the PCR assay resulted in a 283-bp bovine mtcyt-b DNA product.

All primers were synthesized on a DNA synthesizer (Milliigen/Biosearch, a division of Millipore Burlington, MA)

and purified using oligo-pak oligonucleotide purification columns (Glen Research Corporation, Sterling, VA.) as per manufacturer's instructions.

2.4 Polymerase Chain Reaction (PCR):

2.4.1 1st Run: Direct PCR

A stock buffered solution containing 250 µl 10X PCR buffer, 100 µl of $MgCl_2$, 12.5 µl of each dATP, dTTP, dGTP and dCTP was prepared in 1.5 ml eppendorf tube, and double distilled water was added to bring the volume of the stock buffer solution to 1.5 ml. The primers were used at a concentration of 20 µmole / L which appears to 2 µl. Next, 5.0 µl of the target DNA was added to 42µl of the stock solution in 0.5 ml PCR tubes and mixed by vortexing. This is followed by 1.0 µl of Taq DNA polymerase (Perkin Elmer) which was used at a concentration of 2.5 units. All PCR amplification reactions were carried out in a final volume of 50 µl.

The thermal cycling profiles were as follows: 2-minutes incubation at 95°C, followed by 40 cycles at 94°C for 1 minute, 57°C for 30 sec and 72 °C for 45 sec, and a final incubation at

72°C for 10 minutes. Thermal profiles will be performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ.USA)(Appendix-Fig5). These steps were used in both the preliminary study and in the first stage of the main study.

2.4.2 2nd Run: Nested (nPCR) and Semi-nested PCR:

For the nested and semi-nested PCR assay, 2 µl of the product produced by first primary amplification were transferred to PCR tube containing 2 µl of the internal primers (PSL3 and PSR4) in cases of specific identification of swine mtcyt-b gene or (CSL1 and CSR2) incase of specific identification of bovine mtcyt-b, 45 µl of 10 X PCR buffer mixture and 1.0 µl Taq DNA polymerase. The total volume of the PCR reaction mixture was brought to 50 µl. The thermal cycling profiles were as the same as used in the first amplification step.

2.5.3 Semi-nested PCR

For the semi-nested PCR amplification, 2.0 µl of the primary products produced by CSL1 and CSR2 were transferred to 0.5 ml

PCR tube containing (2 µl of semi-nested primers and; 45 µl of stock PCR buffer and 1µl Taq DNA polymerase was used at a concentration of 5.0 U/µl. The semi-nested pair of primers (CSL1&CSR2) was expected to amplify 284 bp PCR products, internal to the annealing sites of primers RSL1 and CSR2. All PCR amplifications were carried out in a final volume of 50 µl. The thermal cycling profiles were as follows: 2 minutes incubation at 95° C, followed by 30 cycles of 94°C for 1 minute, 57°C for 30 sec and 72°C for 45 sec, and a final incubation at 72°C for 10 minutes. Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ.)

2-6 Visualization of PCR Products

All PCR amplification product samples were visualized using agarose gel electrophoresis. The 10X Tris borate EDTA (TBE) buffer was diluted to 1X solution which was used to prepare 1.0 % agarose gels and as running buffer in electrophoresis after it was stained with ethidium bromide as0.5 µg/ml.

15 µl from each PCR reaction containing amplified product

was loaded onto gels of 1.0 % SeaKem agarose (FMC Bioproduct, Rockland ME) and was electrophoresed (Appendix-Fig.6).

The results were visualized under UV light transluminator (Appendix-Fig.7). The results were then photographed by gel documentation system (Appendix- Fig.8)

CHAPTER THREE

RESULTS

3-1 Preliminary study

3-1-1 Specificity

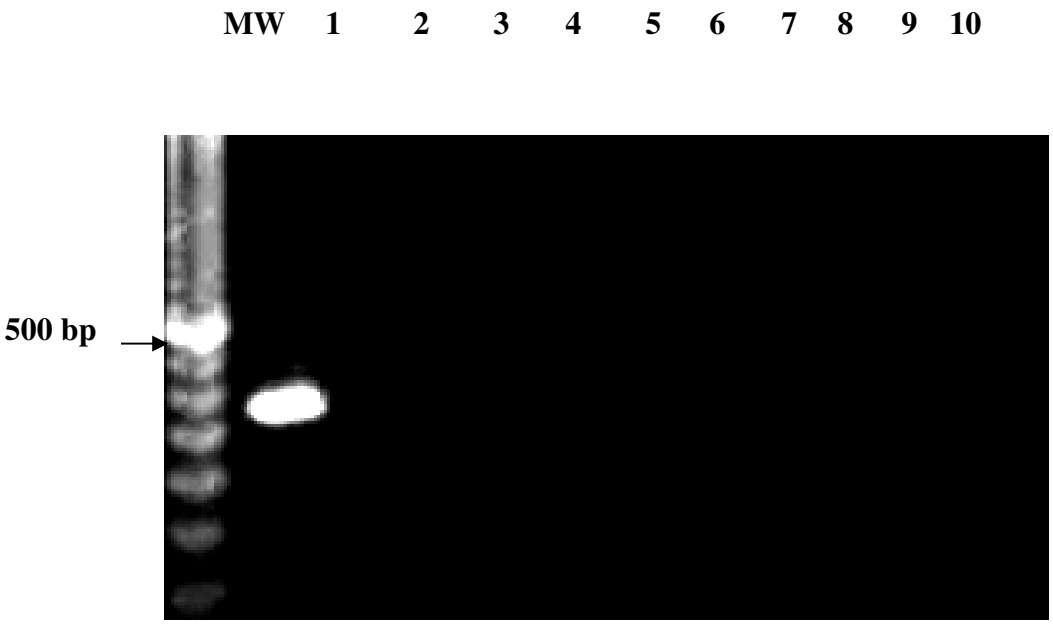
In the preliminary study of the swine specific mtcyt-b gene PCR amplification, the pair of primers (PSL1&PSR) was used. 1.0 pg target DNA produced 525 bp PCR products as clearly shown in (Fig.9). The other target DNAs included cattle, sheep, goat, camel, deer, horse, donkey, chicken and fish respectively, gave negative results to amplification with swine specific mtcyt-b gene. The laboratory specificity in this case was measured as follows:

$$\text{Laboratory Specificity} = \frac{\text{Number of samples that test PCR negative}}{\text{Total number of negative samples}} = {}^{9}/_{9} \times 100 = \underline{\mathbf{100\%}}$$

3-1-2 Sensitivity

On the other hand, the sensitivity was tested for the same reaction using 10 fold serial dilutions for the initial concentration 1ng/ µl. The results of PCR amplification using

Fig. (9)



Specificity of the PCR assay for the detection of the swine specific mtcyt-b gene.

Lane MW: molecular weight marker (100 bp DNA ladder); Lane 1: 1.0 pg of swine DNA(positive control); Lane 2-10 : 1.0 ng of mtcyt-b gene of cattle, sheep, goat, camel, deer, horse, donkey, chicken and fish respectively.

The pair of primers (PSL1&PSR) indicated that this reaction can amplify as far as 100fg of swine mtcyt-b gene. The size of each product was 525bp and these results are shown in (Fig.10). The measured sensitivity below showed the high percentage as follows:

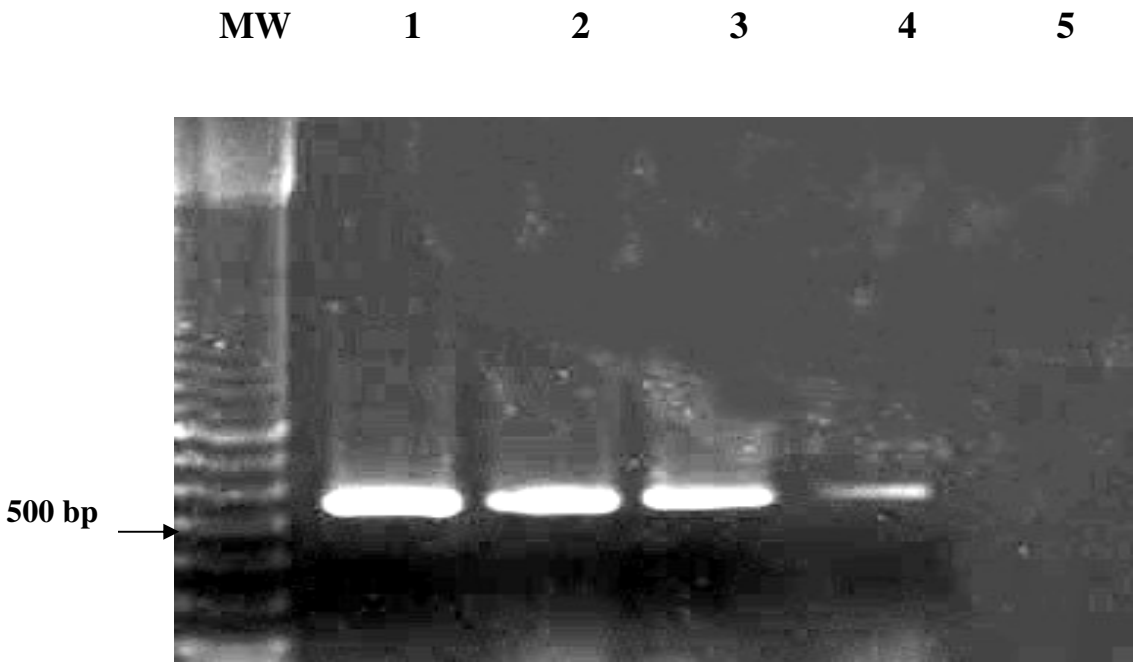
$$\text{Laboratory Sensitivity} = \frac{\text{Number of samples that test PCR positive}}{\text{Total number of positive samples}} = {}^{4}/_{5} \times 100 = \underline{\mathbf{80\%}}$$

3-1-3 Processed Pork

Also the technique was applied to the processed pork such as cooked, smoked and marinated pork. (Fig.11) shows the results in which PCR detected swine mtcyt-b gene in all treated pork.

$$\text{Laboratory Sensitivity} = \frac{\text{Number of samples that test PCR positive}}{\text{Total number of positive samples}} = {}^{4}/_{4} \times 100 = \underline{\mathbf{100\%}}$$

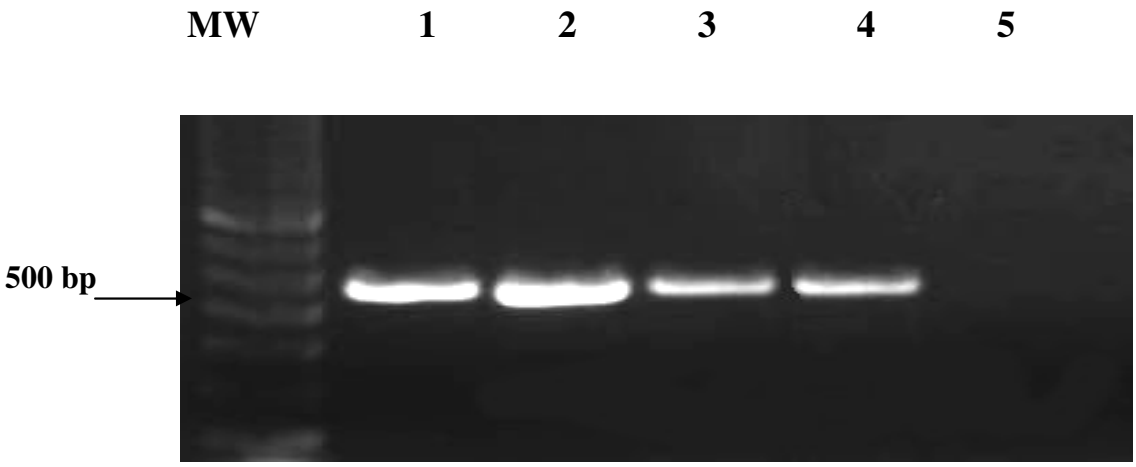
Fig. (10)



Sensitivity of the PCR assay for the detection of the swine specific mtcyt-b gene.

Lane MW: molecular weight marker. Lanes1-5: 525bp PCR products amplified from swine DNA at concentrations of 1ng, 100pg, 10pg, 1pg, 100fg respectively.

Fig. (11)



Detection of the specific 525 bp PCR product from processed pork.

Lane MW: molecular weight marker; lane 1: 1 pg swine DNA (positive control); lane 2: marinated ham; lane 3: microwaved ham for 15 minutes; lane 4: smoked ham; lane 5: sheep DNA (negative control).

3-2 Main study

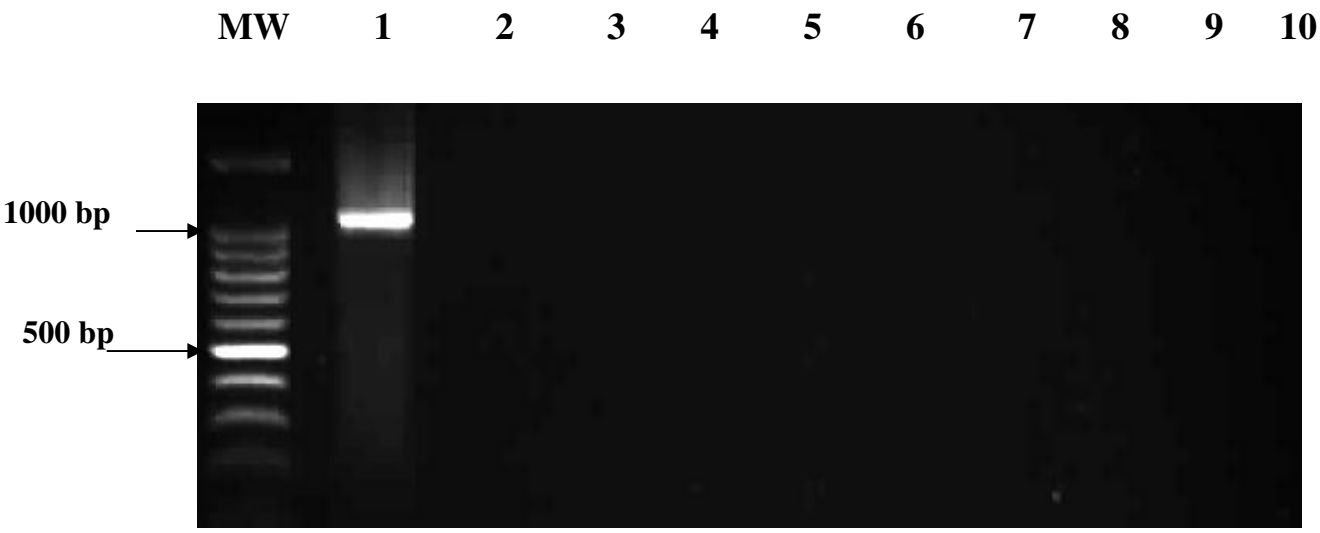
3-2-1 Specificity

In the main study the number of samples was increased, another pair of primers was used and the nested amplification was also applied using a third pair of primers.

When primers (PSL1&PSR2) were used for specific amplification of swine mtcyt-b gene, they gave the same specificity result as in the preliminary study. This can obviously be noticed in (Fig.12). Amplification product was not detected from DNA of other animal species including either ruminants as sheep, goat, cattle, deer, camel or nonruminants like horse, donkey, chicken and fish. The size of the PCR product in this case is 1055bp and the calculations of the specificity are:

$$\text{Laboratory Specificity} = \frac{\text{Number of samples that test PCR negative}}{\text{Total number of negative samples}} = {}^{9}/_{9} \times 100 = \underline{\mathbf{100\%}}$$

Fig. (12)



Specificity of the polymerase chain reaction for specific identification of swine DNA using outer primers (PSL1 and PSR2).

Lane MW: molecular weight marker; Lane 1: 1 pg swine DNA (positive control); Lane 2-10: DNA extracted from sheep, goat, cattle, camels, deer, horses, donkeys, chicken and fish; respectively.

3-2-2 Sensitivity

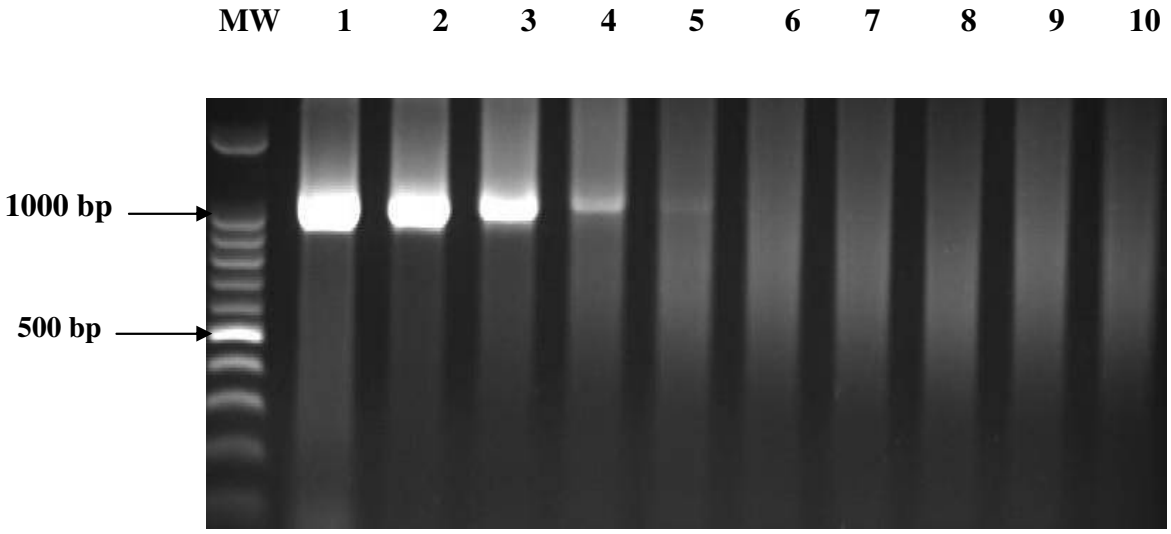
The sensitivity of the technique was detected using 10 fold serial dilutions for the initial concentration 1ng/ µl. The pair of primers (PSL1&PSR2) was used in this step which later produced 1055bp PCR products. The results revealed that as far as 100pg can be detected as clearly shown in (Fig.13). When the nested amplification was used for the same products, the sensitivity was obviously increased. The inner primers (PSL3&PLS4) increased the ability of the gene to be amplified. So the swine mtcyt-b gene can be detected as far as 0.001 fg which is equal to one copy of the gene (Fig.14). Application of the technique on different animals DNA samples showed the same results (Fig.15, Fig.16). The laboratory sensitivity can be calculated as follows:

$$\text{Laboratory Sensitivity} = \frac{\text{Number of samples that test PCR positive}}{\text{Total number of positive samples}} = {}^{9}/_{9} \times 100 = \underline{\mathbf{100\%}}$$

Or

$$= {}^{13}/_{13} \times 100 = \underline{\mathbf{100\%}}$$

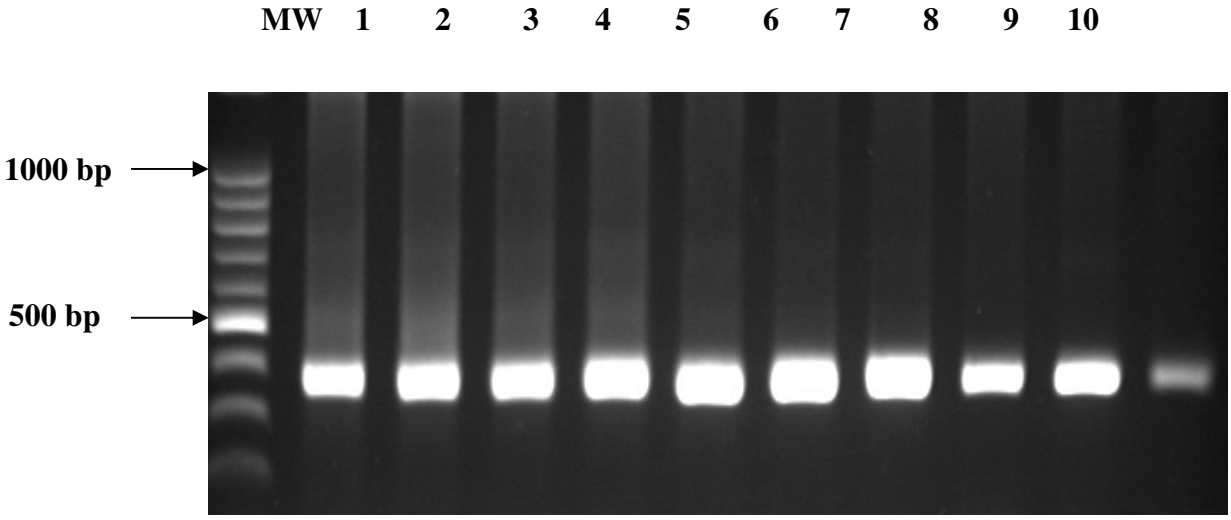
Fig. (13)



Sensitivity of the PCR assay for the detection of the swine-specific 1055 bp PCR product using primers (PSL1 and PSR2).

.Lane MW: molecular weight marker (100 bp ladder); lanes1-10: swine DNA at concentrations of 1ng, 100pg, 10pg, 1pg, 100fg, 10fg, 1fg, 0.1fg, o.o1 fg and 0.001 fg, respectively.

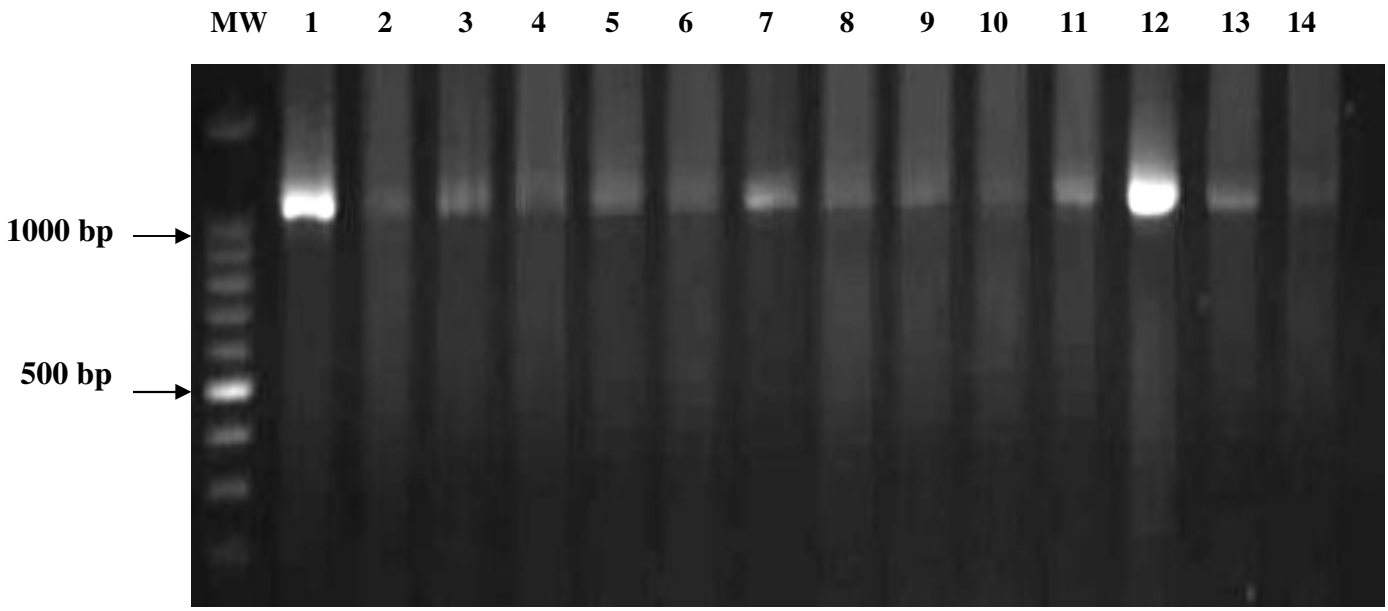
Fig. (14)



Nested amplification of the 361 bp PCR products from the primary PCR product.

Lane M: molecular weight marker; lanes1-10: swine DNA at concentrations of 1ng, 100,pg, 10pg, 1pg, 100fg, 10 fg, 1fg, 0.1fg, o.o1 fg and 0.001 fg , respectively.

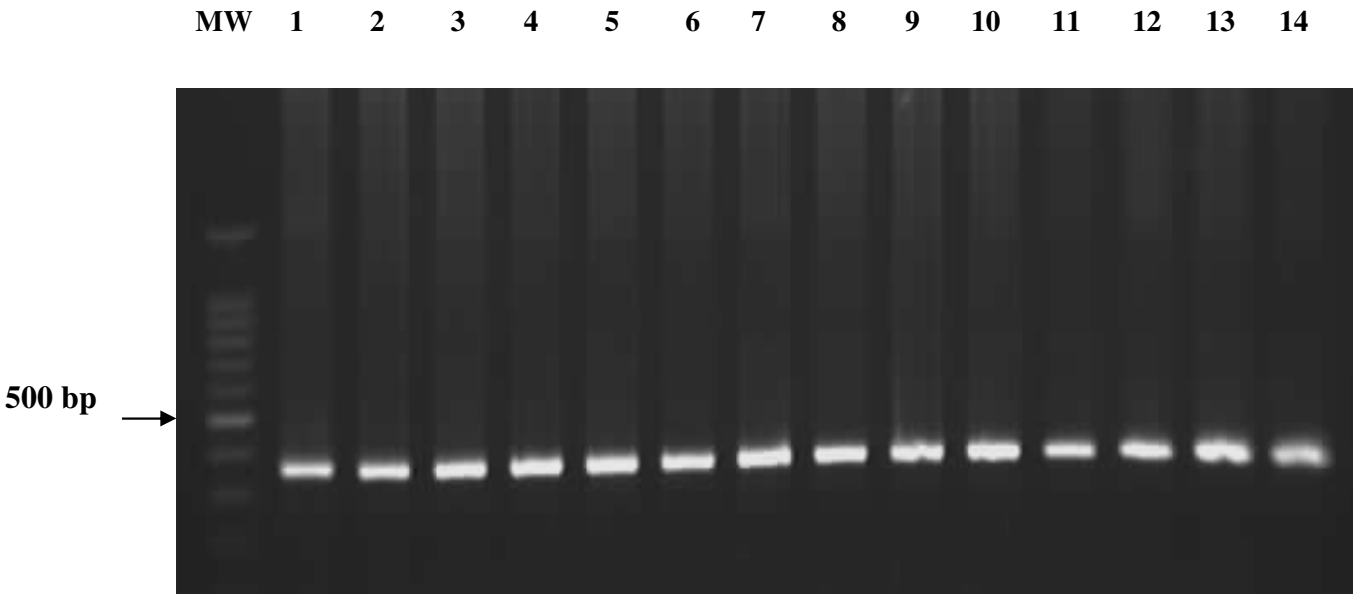
Fig. (15)



Detection of the specific1055 bp PCR product

Lane MW: molecular weight marker; lane 1: swine DNA (positive control); lane 2-14: 13 swine DNA collected from 13 different animals.

Fig. (16)



nPCR to detect the specific361 bp PCR product

Lane MW: molecular weight marker; lane 1: swine DNA (positive control); lane 2-14: 13 swine DNA collected from 13 different animals.

3-2-3 Processed Food and commercial feed:

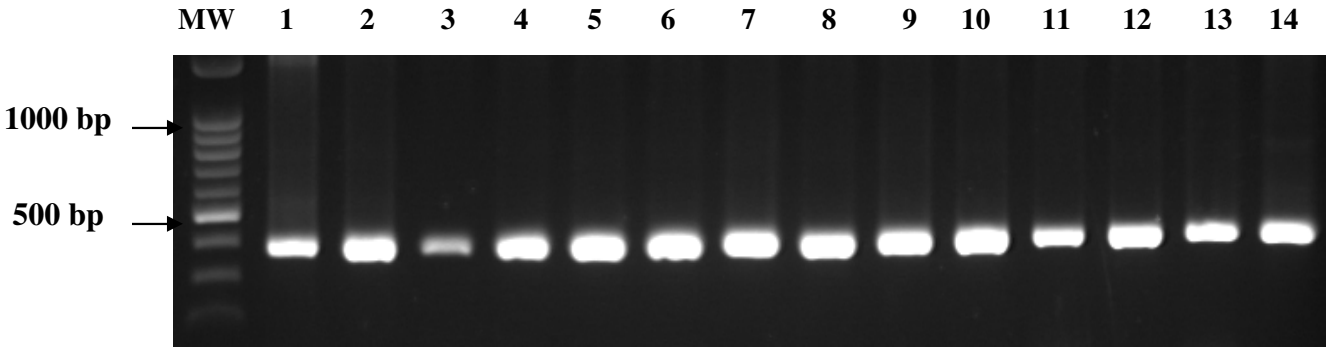
Then directly nPCR was applied on processed food to detect the swine specific mtcyt-b gene. This included marinated ham, microwaved ham for 15 minutes, smoked ham, boiled ham supernatant, boiled ham sediment, frozen ham, cooked luncheon, boiled pork, boiled luncheon sediment, frozen pork, canned pork and pig fat (tallow). As clearly shown in (Fig.17), the 361 bp products from swine mtcyt-b gene was successfully amplified from food which processed with common used treatments and processing such as marinating, smoking, boiling, cooking, freezing and canning.

$$\text{Laboratory Sensitivity} = \frac{\text{Number of samples that test PCR positive}}{\text{Total number of positive samples}} = {}^{14}/_{14} \times 100 = \underline{\mathbf{100\%}}$$

In commercial feed such as pets' food and poultry feed revealed the same results of amplification of swine mtcyt-b gene using nPCR (Fig.18).

$$\text{Laboratory Sensitivity} = \frac{\text{Number of samples that test PCR positive}}{\text{Total number of positive samples}} = {}^{5}/_{5} \times 100 = \underline{\mathbf{100\%}}$$

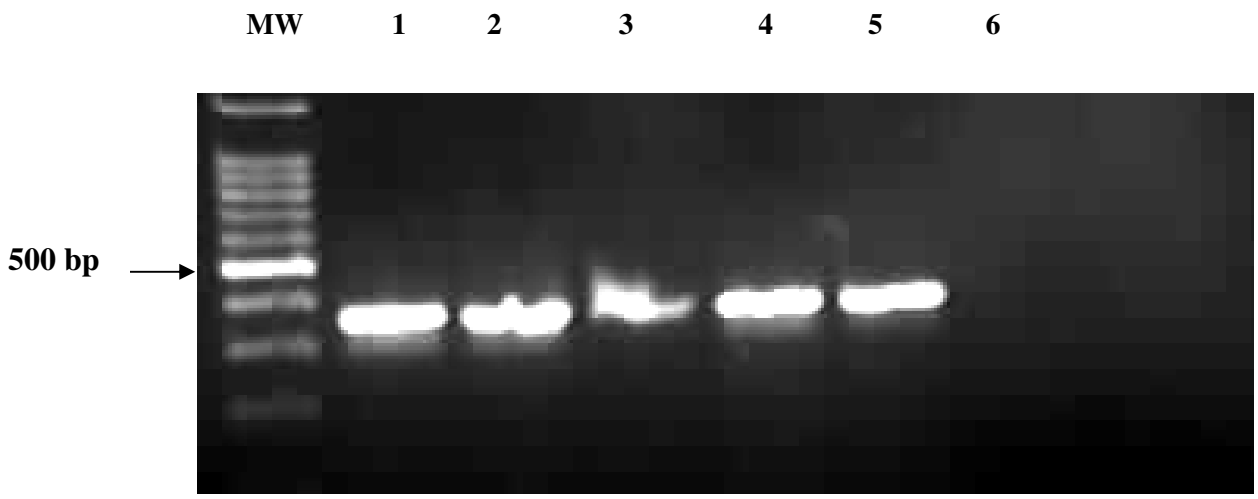
Fig. (17)



nPCR for direct detection of the specific 361 bp PCR product from swine-derived product in processed food and in animal feed concentrates.

Lane 1: 1 pg swine DNA (positive control); lane 2: marinated ham; Lane 3: microwaved ham for 15 minutes; lane 4: smoked ham; lane 5: boiled ham supernatant; Lane 6: boiled ham sediment; lane 7-8: frozen ham; lane 9: cooked luncheon; lane 10: boiled pork; Lane 11: boiled luncheon sediment; lane 12: frozen pork; Lane 13: canned pork; lane 14: tallow.

Fig. (18)



nPCR for direct detection of 361 bp PCR product from swine-derived product in commercial animal feed concentrates.

Lane 1: swine blood (positive control); lane 2-3: pets food; Lane 4-5; dog food; Lane 6-7: bovine DNA (negative controls).

3-3 Semi nested PCR:

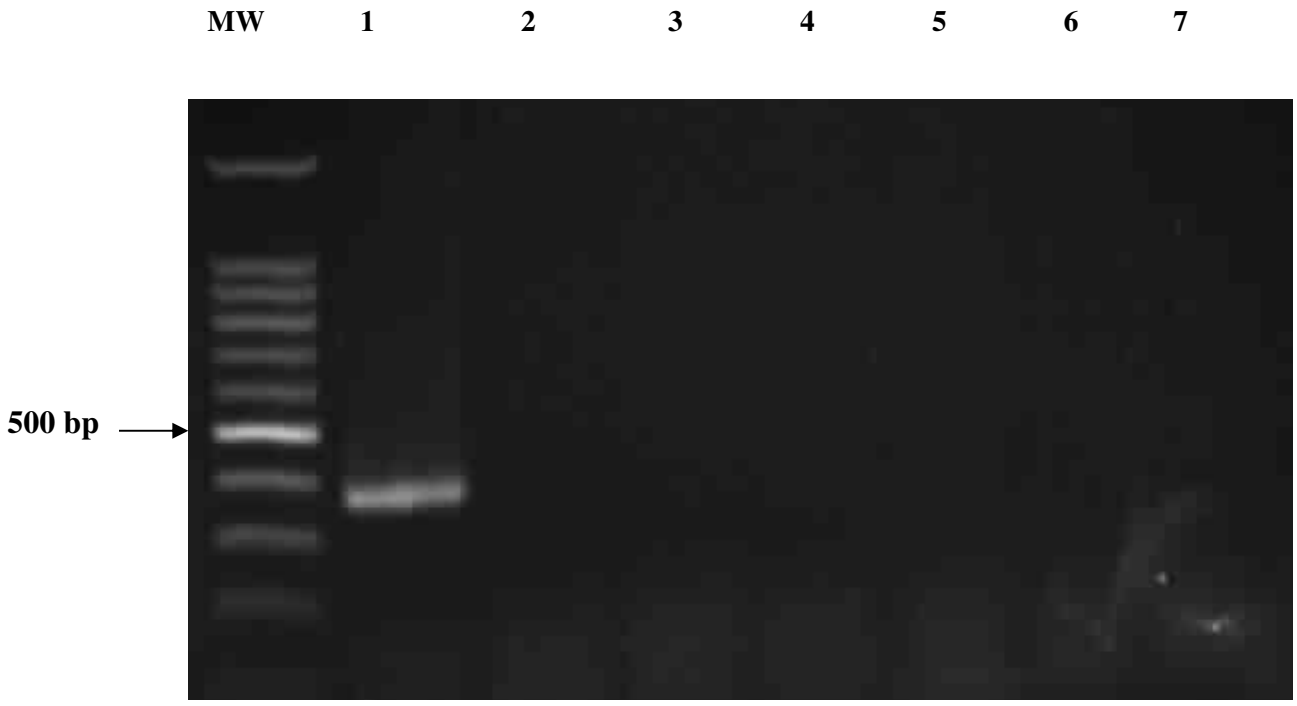
This technique- which is similar to nPCR- was applied to bovine (cattle) mtcyt-b gene. The technique gave the same results as nPCR in swine mtcyt-b and this is clearly shown in (Fig.19-Fig.23).

$$\text{Laboratory Sensitivity} = \frac{\text{Number of samples that test PCR positive}}{\text{Total number of positive samples}} = {}^{7}/_{7} \times 100 = \underline{\mathbf{100\%}}$$

Or

$$= {}^{14}/_{14} \times 100 = \underline{\mathbf{100\%}}$$

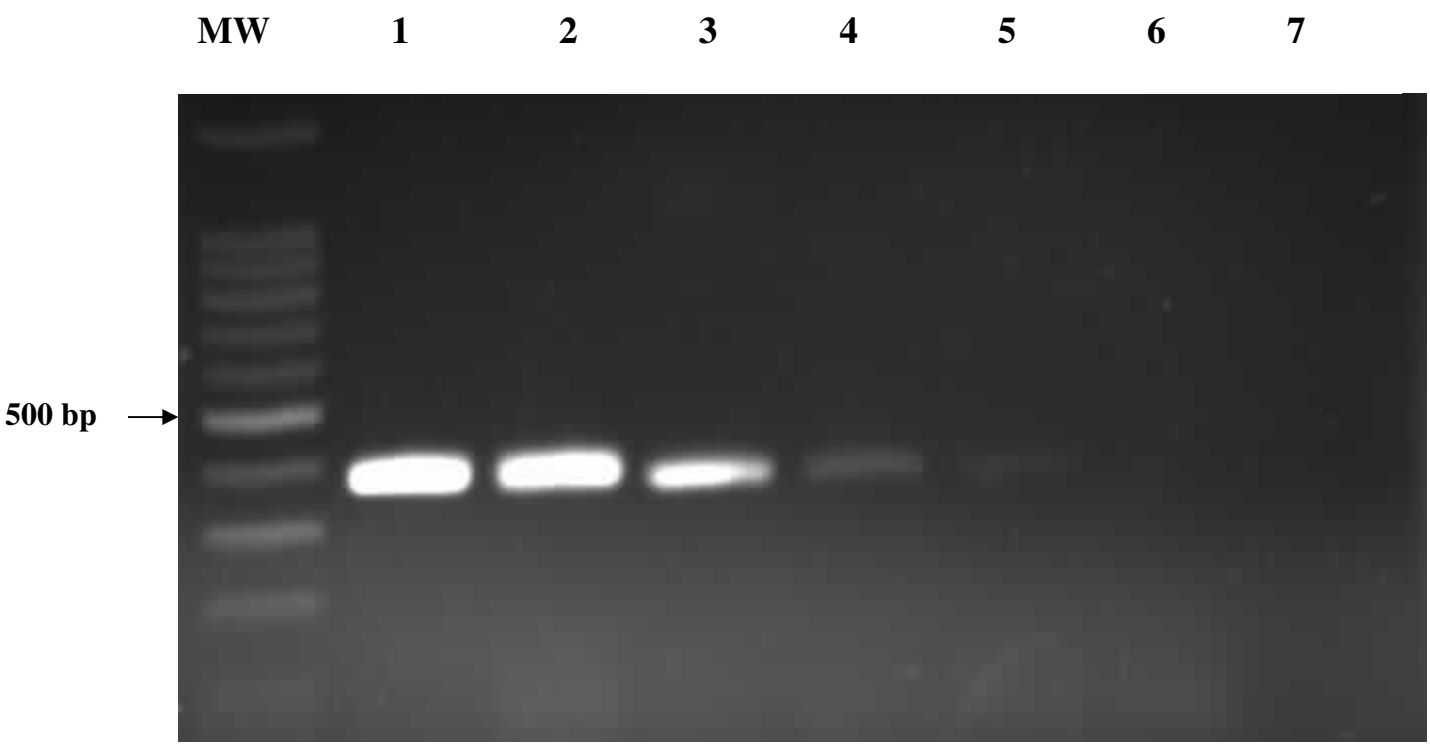
Fig. (19)



Specificity of the polymerase chain reaction for specific identification of bovine DNA using primers (CSL1&GSR2).

Lane MW: molecular weight marker; Lane 1: 1.0 ng of bovine mtcyt-b DNA (positive control); Lane 2-4: 1.0 ng of ruminant mtcyt-b DNA extracted from other ruminant species including sheep, goat and deer respectively. Lane 5-7: 1.0 ng of non ruminant mtcyt-b DNA extracted from other ruminant species including horse, donkey and pig respectively.

Fig. (20)

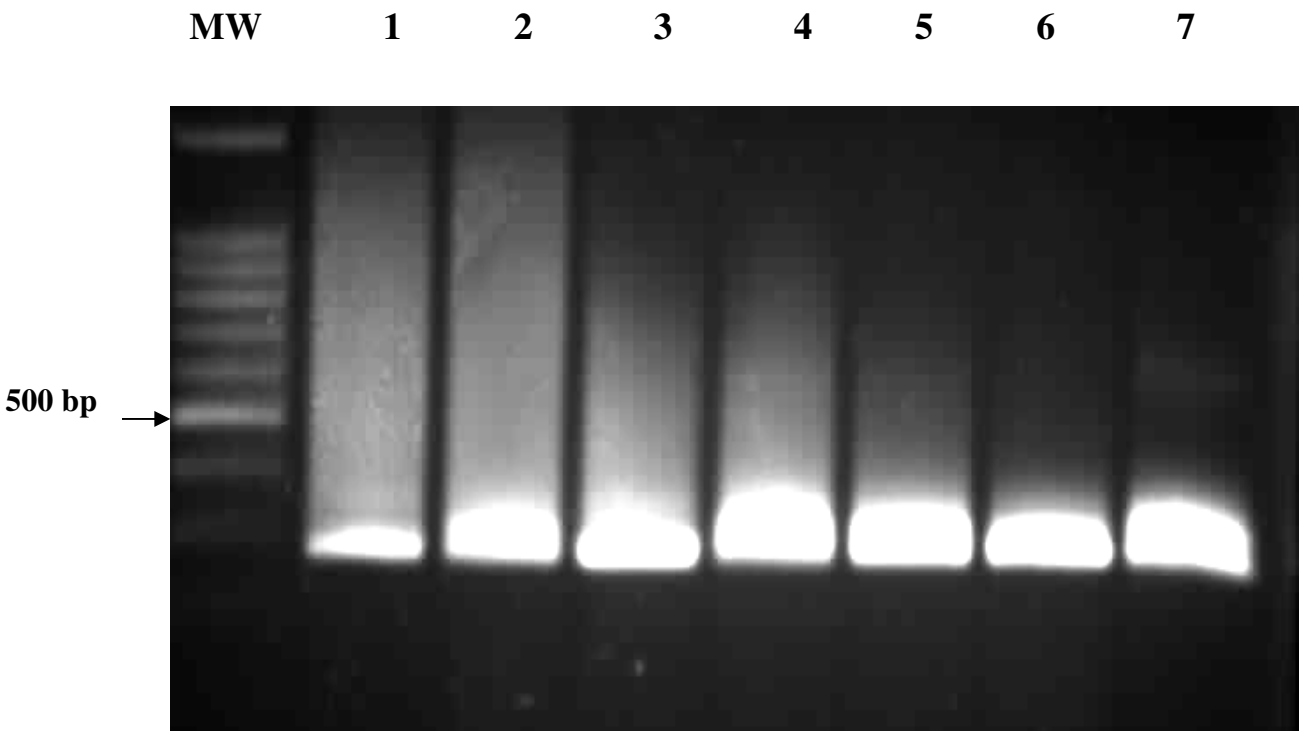


Sensitivity of the PCR assay for the detection of the bovine-specific 365 bp PCR product, using primers RSL1 and CSR2.

Visualization of the 365 bp PCR product on ethidium bromide-stained agarose gel from 100fg of bovine DNA.

Lane MW: molecular weight marker (100 bp ladder); lanes1-7: bovine DNA at concentrations of 1ng, 100pg, 10pg, 1pg, 100fg and 10 fg, respectively.

Fig. (21)

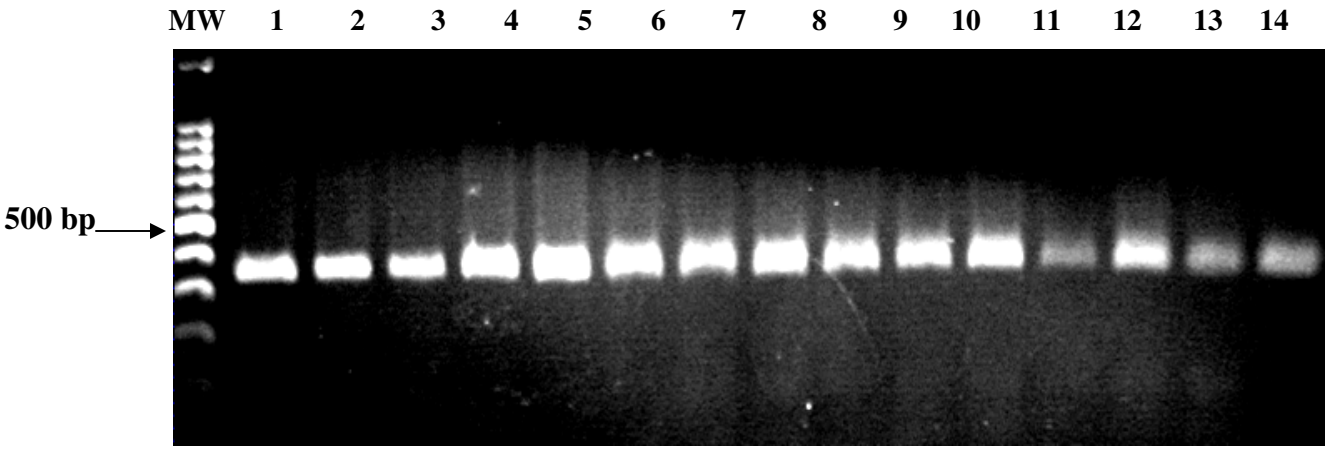


Semi-nested amplification of the 361 bp PCR products from the primary PCR product.

The nested PCR amplification detected as little as 0.001 fg of swine DNA.

Lane MW: molecular weight marker (100 bp ladder); lanes1-7: bovine DNA at concentrations of 1ng, 100pg, 10pg, 1pg, 100fg and 10 fg, respectively.

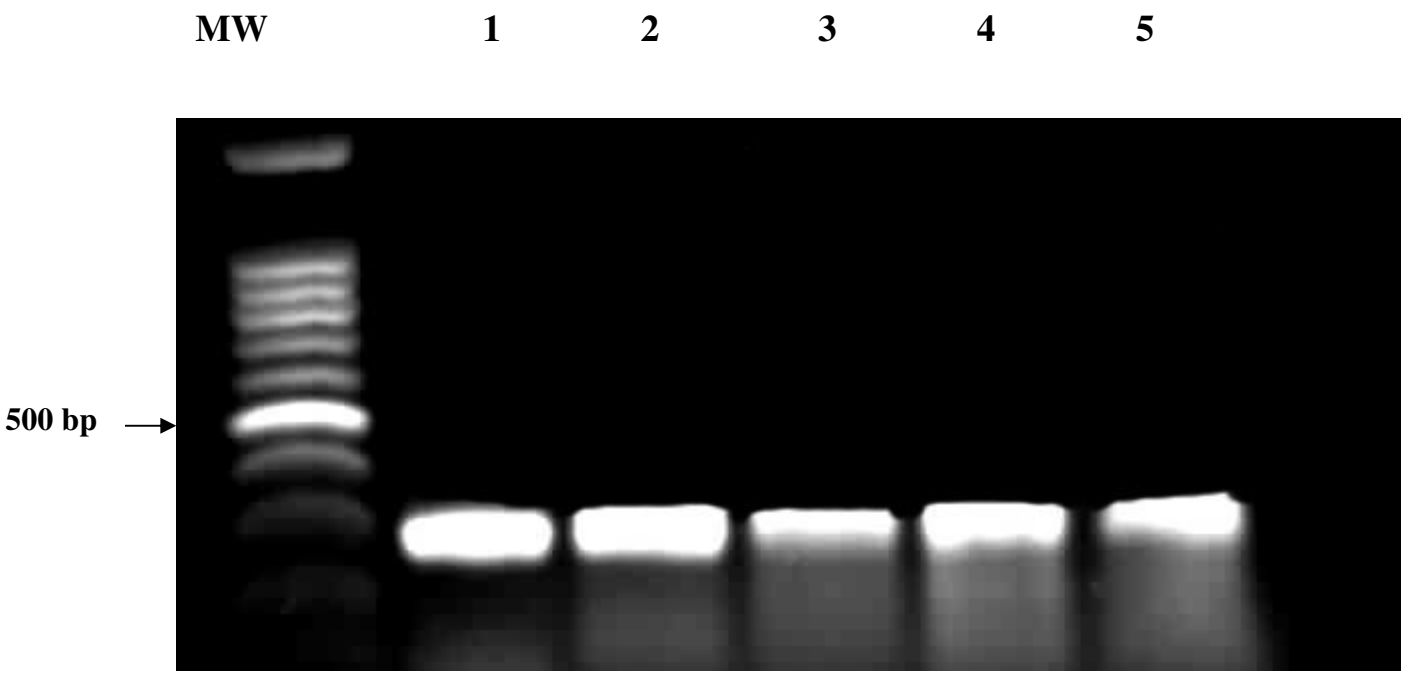
Fig. (22)



Ampilification of the PCR assay for the detection of the bovine-specific 365 bp PCR product, using primers RSL1 and CSR2.

Lane MW: molecular weight marker; Lane 1: 1.0 ng of bovine mtcyt-b DNA (positive control); Lane 2-15: 14 different samples.

Fig. (23)



Semi-nested PCR to detect bovine DNA in animal feed concentrates. Lane 1: Bovine DNA (positive control), Lane 2and 3: candies containing bovine gelatin, Lane 4: dog food; Lane 5: commercial cattle feed concentrates.

CHAPTER FOUR

DISCUSSION

In this study, the Polymerase Chain Reaction was used in a variety of protocols. The technique depends on the DNA gene extracted and its concentration. The total molecular weight of mitochondrial genome has been detected to be 11.44×106 Da, and 10 fg of mtcyt-b DNA corresponds to 1000 copies of this gene (Aradaib *et al*., 1998b). From the preliminary and main experiments of this study we can prove that the protocols used in this study are very specific and sensitive while both 525bp and 1055 bp PCR products (from preliminary and main experiments, respectively) were detected from 1.0 pg of swine mtcyt-b gene but not from as far as 1.0 ng mtcyt-b gene of other animals including cattle, sheep, goat, deer, camel, horse, donkey, chicken and fish. This gave 100% of laboratory sensitivity. Further, the sensitivity of these primers was studied. The PCR assay detected as far as 100 fg in the preliminary study and 100 pg in the main study giving laboratory sensitivity reaching 80% and 100% respectively. According to these results,

we can suggest that the initial PCR amplification can be used for tentative diagnosis of presence of swine derived products.

In the main study, the nested PCR (nPCR) increases the sensitivity of the primers with the possibility of amplification of target sequence inside the initial amplification targets. So the sensitivity was increased to detect as far as 0.001 fg of swine specific mtcyt-b gene which correspondss to 1 copy of this gene. Also the laboratory sensitivity increased from 80% to 100%.

Similar findings were obtained when a Semi-nested PCR-a technique- which is similar to nPCR-was applied to bovine (cattle) mtcyt-b gene which states a further prove of specificity and sensitivity of PCR protocols.

These protocols represent a possible alternative to immunological methods (Allman et al., 1993 Mayer et al., 1994, Aradaib et al, 1998a-b, Aradaib et al., 2001, Aradaib et al., 2004) while the antigenes used in those methods are proteins in nature and much affected by physical and chemical factors such as heat and lysing enzymes.

We find that the nested and semi-nested PCR assays are simple and rapid. They spent only five hours in both amplification and

need no incubation for hours like the analysis of the PCR fragments with endonuclease enzymes to detect restriction fragment length polymorphisms (RFLPs) (Rodriguez et al 2004, Allman et al., 1993, Mayers et al., 2004, Lanzilao et al, 2005).This technique is expensive and time consuming. Also the techniques applied in this study need no sophisticated laboratory equipments such like DNA sequencers and hybridization facilities as found in direct sequencing and digestion of PCR products with endonucleases (Mayer et al., 2004)

Conclusion and Recommendations:

Besides the previous studies which detected ruminants or sheep or goat meat (Aradaib et al., 1998a-b, Aradaib et al, 2001), the nested and semi-nested PCR assays used in this study are simple, rapid and cheap methods for detection and specific identification of animal-derived products in animal concentrates and processed food compared with those tedious, laborious, cumbersome, expensive and time consuming techniques. Furthermore, the techniques used in this study would be advantageous in variety of conditions including comparative

genomics, investigative forensics and can be recommended in quality control departments in order to support policies and regulation of import/export of animal derived products.

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Appendix

1. **(1M) Trisodium Citrate (mw:149.01)(Tris)Buffer:-**

   To prepare 100 ml of (1M) Trisodium Citrate Buffer, 14.901 gm added to 100 ml of D.W. and pH adjusted to pH 8.0.

2. **(0.1M) Tris Buffer:-**

   To prepare 100 ml of (0.1M) Trisodium Citrate, 10 ml of (1M) Trisodium Citrate Buffer added to 90 ml of D.W.

3. **(3M)Sodium Acetate(MW:82.03)Buffer:-**

   To prepare 100 ml of (3M) Sodium Acetate Buffer, 24.909 gm added to 100 ml of D.W. and pH adjusted to pH 5.0.

4. **(1M) Sod. Acetate Buffer:-**

   To prepare 150 ml of (1M) Sodium Acetate Buffer, 50 ml of (3M) Sodium Acetate Buffer added to 100 ml of D.W.

5. **(0.1M) Sod. Acetate Buffer:-**

To prepare 100 ml of (0.1M) Sodium Acetate Buffer, 10 ml of (1M) Sodium Acetate Buffer added to 90 ml of D.W.

6. **Tris buffered Phenol(56°C):-**

Saturate 50 ml Phenol with 50 ml Tris (0.1 M) water bathed on 56°C for 10 minutes. The supernatant was poured off and added another 50 ml Tris (0.1 M) then water bathed on 56°C for another 10 minutes and this repeated for a third time without pouring off the supernatant and the working solution is stored in the refrigerator.

7. **Sod. acetate buffered Phenol(56°C):-**

Saturate 50 ml Phenol with 50 ml sod. acetate (0.1 M) water bathed on 56°C for 10 minutes supernatant was poured off and another 50 ml sod. acetate (0.1 M) were added, then water bathed on 56°C for another 10 minutes and this repeated for a third time without pouring off the

supernatant and the working solution is stored in the refrigerator.

8. 10% Sod. dodecyl sulphate (SDS):-

10 gms of (SDS) were dissolved in 100 ml of D.W.

9. Stock Ethidium Bromide Solution:-

10 mg of Ethidium Bromide were added to 1ml of D.W.

10. 1 X TBE Buffer:-

To Prepare 500 ml of 1 X TBE Buffer, 50 ml of the stock 10 X TBE Buffer, pH8.3 (Promega Quality Biochemicals, Madison, WI 53711-5399, USA) added to 450ml of D.W.

11. Running Electrophoresis Running Buffer:-

To prepare 500 ml of Electrophoresis Running Buffer, 25 µl of the stock ethidium bromide solution added to 500 ml of 1 X TBE Buffer (ethidium bromide reaches 0.5µg/ml of buffer).

Fig. (1): Bench Centrifuge

Hettich Zentrifugen, Beta 20, D-785320, Tuttiligen, Germany

Fig. (2): Vortex Machine

Janke &Kunkel,GmbHu.CoKG,

Germany

Fig. (3): Microcentrifuge

Hettich Zentrifugen, 12-24, Tuttiligen, Germany

Fig. (4): QIAamp Blood Kit

QIAGEN Inc. Chatsworth, Canada

(1) 2 ml collecton tube, (2) QIAamp spin column, (3) LA Lysis Buffer, (4) Elution Buffer, (5) Washing buffer1, (6) washing buffer 2.

Fig. (5): Theralcycler

Techne, Princeton, NJ.USA

Fig. (6): Gel Electrophoresis Set

Fig. (7): Transluminator (U.V. light source)

Fotodyne, Inco. USA

Fig. (8): Gel Documentation System

Uvitec, USA